PRESENCE OF VIRULENCE MARKERS IN POLISH STREPTOCOCCUS SUIS FIELD ISOLATES

ANNA SZCZOTKA AND IWONA MARKOWSKA-DANIEL

Department of Swine Diseases, National Veterinary Research Institute, 24-100 Pulawy, Poland
anna.szczotka@piwet.pulawy.pl

Received for publication August 21, 2008

Abstract

The objective of this study was to detect proteins related with the virulence of *Streptococcus suis* (*S. suis*): muramidase-released protein (MRP), extracellular factor (EF), and suilysin (SLY), using the Western blot procedure. The strains were isolated in Poland from pigs with clinical symptoms and pathological lesions of streptococcosis. Eleven phenotypes were identified among the tested strains of serotype 2. Half of them (43) produced all three proteins, i.e. 40 strains (46.51%) produced MRP+ EF+ SLY+ and three isolates (3.49%) produced MRP, EF*, and SLY. Thirteen strains (15.12%) produced two proteins. Twenty-three (26.75%) strains synthesized only one protein. No proteins were detected in seven (8.13%) strains.

*S. suis* isolates belonging to serotypes other than serotype 2 represented six phenotypes. There was no isolate synthesising all the three potential virulence factors in this group. These results show that MRP, EF, and SLY are produced in 50% of the strains. Because these isolates originate from diseased pigs, also other factors than MRP, EF, and SLY must be involved in *S. suis* virulence.

Key words: *Streptococcus suis*, virulence, muramidase-released protein, extracellular factor, suilysin.

*Streptococcus suis* (*S. suis*) is recognised as a very common and significant pathogen of swine. *S. suis* infection may be the cause of meningitis, polyarthritis, polyserositis, endocarditis, septicaemia, and sudden death, resulting in significant economic losses in pig farms (9). This pathogen is also considered as a dangerous for humans (10).

Strains isolated from pigs showed different levels of virulence - from highly virulent to non-pathogenic (20, 21). Although several factors until now have been studied, no universal virulence marker of *S. suis* has been identified (9). The following proteins are considered the most probable virulence factors: the 136 kDa muramidase-released protein (MRP), 110 kDa extracellular factor (EF) and 54-65 kDa suilysin (SLY) (9, 12). Based on the presence of MRP and EF in *S. suis* strains, three main phenotypes were described: MRP+EF+, are considered as virulent strains; MRP+EF-, are considered as strains associated with slight pathological changes; and MRP-EF-, are considered as potentially avirulent strains (19). However, it has been demonstrated by Smith et al., (16) that streptococci of phenotypes: MRP-EF-, MRP+EF-, MRP-EF+, and MRP+EF* were able to induce clinical infection. Variants of MRP and EF proteins showing different levels of electrophoretic mobility, MRP* (molecular weight, MW >136 kDa), MRP* (MW <136 kDa), and EF* (MW > 110 kDa) have also been detected (7, 19).

The aim of the study was to identify MRP, EF, and SLY among Polish field *S. suis* isolates.

Material and Methods

Bacterial strains. The study was performed on the strains isolated from pigs showing clinical signs and gross lesions typical of streptococcosis. One hundred and seven strains were analysed. These isolates were initially identified as members of *S. suis* species on the basis of biochemical profiles (18) and PCR results (13). In this group, 86 (80.37%) strains belonged to serotype 2, 17 (15.89%) to serotype 1/2, and four (3.74%) to serotype 1.

Media. Bacteria were grown overnight on Columbia blood agar base supplemented with 5% of sheep blood and in Todd-Hewitt broth, at 37°C with 5% CO₂.

Preparation of culture supernatants. The 18-h-old cultures of each bacterial strain were prepared. A few colonies were transferred into broth and incubated for 18 h (conditions as mentioned above). The cultures were centrifuged (10 min, RT, 4,000 rpm), and then the supernatants were filtered through syringe filters (0.22 µm). The filtrates were condensed ten times in a vacuum centrifuge, mixed with reducing buffer, vortexed, and then boiled for 5 min in a water bath. After spinning
down, the supernatants were stored at -20°C until their use.

**Electrophoresis.** Cell fractions were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis under reducing conditions in Mini Protean 3 apparatus (Bio-Rad). For the detection of MRP and EF proteins, 4.5% stacking gel and 5% separating gel were prepared. The detection of suilysin was conducted in 5% stacking gel and 7.5% concentrating gel. The gel was loaded with 6 µl of molecular mass ruler (Precision Plus Protein Standards, Bio-Rad), negative and positive controls, and tested samples. As positive controls, strains producing all the tested proteins or their variants were used. Electrophoresis was conducted at 100 V until the samples reached stacking gel, and then at 200 V.

**Staining.** The gels were stained in Coomassie blue for 4 h at RT with agitation, and then incubated for 4 h in methanol with acetic acid (RT).

**Transfer.** The separated proteins were then transferred to a nitrocellulose membrane in Mini Trans apparatus (Kucharczyk, Poland) for 1 h at 100 V, or alternatively for 18 h at 30 V.

**Blocking.** The membranes were incubated with agitation for 2 h in blocking buffer (2% milk in TRIS-NaCl) at RT.

**Immunoblot.** After blocking, the membranes were washed three times in TRIS-NaCl for 3 min, and then incubated for 2 h with the specific antibodies. For MRP polyclonal rabbit antiserum (kindly provided by Dr Henk Wisselink, DLO-Central Veterinary Institute, the Netherlands), a dilution of 1:6,000, was used. To identify SLY, polyclonal rabbit antiserum (kindly provided by Dr Marcelo Gottschalk, University of Montreal, Canada) diluted 1:8,000 was applied. After washing three times in TRIS-NaCl, the membranes were incubated for 1 h with the conjugate. For MRP and EF, goat anti-rabbit immunoglobulin conjugated to peroxidase (Jackson, USA) and for SLY – goat anti-mouse immunoglobulin, conjugated to peroxidase (Jackson, USA) were used. The conjugates were diluted at 1:1,500 in a blocking buffer. All the incubations were conducted with agitation at RT.

**Detection.** The membranes were washed five times and stained for approximately for 15 min in the mixture of 30% hydrogen peroxide in TRIS-NaCl with chloronaphthol in ice-cold methanol.

The data were analysed using the GelDoc system (Bio-Rad) with QuantityOne software (Bio-Rad).

**Results**

The result of the examination was interpreted as positive if a product of an expected molecular mass was detected on the membrane. Fig. 1 shows the electrophoretical separation of proteins produced by S. suis.

In Figs 2 and 3, an example of EF and SLY detection, respectively, is presented.

![Fig. 1. Electrophoretic separation of S. suis proteins.](image-url)
**Fig. 2.** Identification of EF on nitrocellulose membrane in Western blot. Lines: M – molecular mass standard, C(-) – negative control, C(+) – positive control, C(v) – molecular mass variant control, 1-6 – tested strains.

<table>
<thead>
<tr>
<th></th>
<th>C(-)</th>
<th>C(+)</th>
<th>C(v)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
</table>

**Fig. 3.** Identification of suilysin on nitrocellulose membrane in Western blot. Lines: C(+) – positive control, C(-) – negative control, M – molecular mass standard, 1-7 – tested strains.

|    | C(+) | C(-) | M   | 1   | 2   | 3   | 4   | 5   | 6   | 7   |
Table 1
Prevalence of MRP, EF and suilysin in Polish serotype 2 of *S. suis* strains

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Number of strains</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRP+ EF+ SLY+</td>
<td>40</td>
<td>46.51</td>
</tr>
<tr>
<td>MRP+ EF- SLY-</td>
<td>16</td>
<td>18.61</td>
</tr>
<tr>
<td>MRP- EF- SLY-</td>
<td>7</td>
<td>8.14</td>
</tr>
<tr>
<td>MRP- EF+ SLY+</td>
<td>6</td>
<td>6.98</td>
</tr>
<tr>
<td>MRP+ EF- SLY+</td>
<td>4</td>
<td>4.65</td>
</tr>
<tr>
<td>MRP* EF- SLY-</td>
<td>4</td>
<td>4.65</td>
</tr>
<tr>
<td>MRP+ EF* SLY+</td>
<td>3</td>
<td>3.49</td>
</tr>
<tr>
<td>MRP- EF- SLY-</td>
<td>3</td>
<td>3.49</td>
</tr>
<tr>
<td>MRP* EF* SLY-</td>
<td>1</td>
<td>1.16</td>
</tr>
<tr>
<td>MRP- EF- SLY-</td>
<td>1</td>
<td>1.16</td>
</tr>
<tr>
<td>MRP* EF- SLY+</td>
<td>1</td>
<td>1.16</td>
</tr>
<tr>
<td>Total</td>
<td>86</td>
<td>100</td>
</tr>
</tbody>
</table>

In total, 67 (60.36%) isolates produced standard MRP molecular mass. MRP* was present in nine (8.11%) strains and MRP in only one (0.9%). Suilysin was detected in more than half of the examined isolates - i.e. in 59 (53.15%) strains. EF was produced by 49 (44.14%) isolates and EF* by four (3.6%) strains.

Based on the results of the performed examination, 11 phenotypes were identified among the tested strains of serotype 2 (Table 1) and six phenotypes among the tested strains belonged to other serotypes, i.e. 1/2 and 1 (Table 2).

Table 2
Prevalence of 1MRP, EF and suilysin in Polish serotypes 1/2 and 1 of *S. suis* strains

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Phenotype</th>
<th>Number of strains</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2</td>
<td>MRP- EF- SLY-</td>
<td>9</td>
<td>42.86</td>
</tr>
<tr>
<td></td>
<td>MRP* EF- SLY-</td>
<td>3</td>
<td>14.29</td>
</tr>
<tr>
<td></td>
<td>MRP- EF- SLY+</td>
<td>2</td>
<td>9.52</td>
</tr>
<tr>
<td></td>
<td>MRP* EF- SLY+</td>
<td>1</td>
<td>4.76</td>
</tr>
<tr>
<td></td>
<td>MRP+ EF- SLY-</td>
<td>1</td>
<td>4.76</td>
</tr>
<tr>
<td></td>
<td>MRP+ EF- SLY+</td>
<td>1</td>
<td>4.76</td>
</tr>
<tr>
<td>1</td>
<td>MRP- EF- SLY-</td>
<td>3</td>
<td>14.29</td>
</tr>
<tr>
<td></td>
<td>MRP+ EF- SLY-</td>
<td>1</td>
<td>4.76</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>21</td>
<td>100</td>
</tr>
</tbody>
</table>

Among serotype two strains, half of them (43) produced all three proteins, i.e. 40 (46.51%) strains produced standard molecular mass proteins (MRP+EF+SLY+) and three (3.49%) isolates produced MRP, EF*, and SLY. Thirteen (15.12%) strains produced two proteins. Among these isolates, EF and SLY were identified in six strains, MRP and SLY – in four, and three strains had the following phenotypes: MRP and EF*, MRP* and SLY, and MRP and SLY. Twenty-three (26.75%) strains synthesised only one protein. Sixteen (18.60%) isolates produced only MRP. Four strains produced only MRP* and three - EF. No proteins were detected in seven (8.13%) strains.

In the case of *S. suis* isolates belonging to serotypes other than serotype 2, six phenotypes were identified. It should be stressed that there was no isolate synthesising all the three potential virulence factors in this group. Strains producing two proteins were identified only among serotype 1/2 (one isolate was MRP+SLY+ and one was MRP*SLY+). Three isolates of serotype 1/2 produced only MRP*, one isolate produced only SLY, and one - MRP. One isolate of serotype 1 produced MRP.

Discussion

Most studies concerning virulence of *S. suis* indicate that MRP, EF, and SLY may play a significant role in the pathogenesis of streptococcosis (5, 7, 9). These three proteins are widespread among *S. suis* strains capable of developing clinical infection in swine (11, 12).

In our study, we found about 51% of the strains representing serotype 2 and producing MRP, EF, and SLY. For example, in Holland, 77% of serotype 2 strains isolated from diseased pigs had phenotype MRP+EF+ (20). In the United States, 56% of isolates from the central nervous system of diseased pigs produced MRP and EF (7). According to these results, it can be expected that *S. suis* serotype 2 posses some other proteins than MRP and EF that can determine its virulence (7).

In our study, we found about 51% of the strains representing serotype 2 and producing MRP, EF, and SLY. For example, in Holland, 77% of serotype 2 strains isolated from diseased pigs had phenotype MRP+EF+ (20). In the United States, 56% of isolates from the central nervous system of diseased pigs produced MRP and EF (7). According to these results, it can be expected that *S. suis* serotype 2 posses some other proteins than MRP and EF that can determine its virulence (7).

On the basis of the results from the detailed studies conducted by Chatellier *et al.* (3), concerning the presence of MRP, EF, their variants, as well as suilysin among *S. suis* serotype 2 isolates from France, England, Holland, Italy, Canada, United States, and Mexico, nine phenotypes were recognised. In our study, eleven phenotypes of serotype 2 and six of other serotypes were identified. Six phenotypes identified among Polish serotype 2 strains were also detected by Chatellier *et al.* (3). Other phenotypes identified by Chatellier *et al.* (3) were absent in our study. These phenotypes were as follows: MRP+EF+SLY+, MRP-EF+SLY-, MRP*EF+SLY-, MRP-EF*SLY-, MRP*EF+SLY+, and MRP*EF+SLY+. The strains of the phenotypes MRP-EF+SLY+ and MRP-EP+SLY+ were found among Polish strains by Fabisiak *et al.* (4). Isolates of phenotypes identified in our study and absent in Chatellier’s investigations (3) were identified among serotype 2 by Galina *et al.* (6) and Wisselink *et al.* (22). The authors mentioned above...
found the MRP-EF+ phenotype in strains from the central nervous system of diseased pigs and MRP*EF-phenotypes were present in both central nervous system of pigs with clinical symptoms of streptococcosis and in nasal cavities of clinically healthy animals (6), but the authors mentioned above did not include the detection of SLY in their study.

The results of our study as well as the data obtained by other authors indicate that mutants of phenotypes MRP- EF+, MRP+EF-, MRP-EF-, and MRP- EF* are able to induce disease, that means that proteins MRP or MRP* and EF present together are not necessary to develop clinical signs of streptococcosis (16).

The analysis including all the three proteins: MRP, EF, and SLY, conducted in various laboratories around the world, was in most cases limited to isolates of serotype 2, which is considered as the classical virulent type. The studies on other serotypes were performed only in a few laboratories. Galina et al. (7) tested strains belonging to serotypes 1, 3, 4, 7 and 10, isolated from the central nervous system of diseased pigs, but none of these strains produced the above-mentioned proteins. The results of our study correspond to those obtained by Galina et al. (7), because among 70% of tested strains of serotypes other than serotype 2, no virulence-related proteins were identified. In the analysis by Smith et al. (17), which included serotypes 1, 1/2, 4, and 9, among serotype 1 MRP*EF+ and MRP-EF-phenotypes were found. An isolate of serotype 9 that did not produce MRP, EF, or SLY was also detected by Smith et al. (17). The presence of such a phenotype was confirmed in other studies (2, 17, 22). In our study in serotype 1, phenotype producing only MRP was detected, which seems to be quite infrequent.

The comparison of S. suis strains from North America and Europe showed that only 8% of American isolates represented MRP+EF+ phenotype, while in Europe this phenotype was present in about 55% of S. suis strains (8, 14). Suiysin was detected in 66% of European strains and in only 22.5% of American isolates (3). Considering this data, it can be concluded that the role of MRP, EF, and SLY as the only factors involved in virulence of S. suis seems to be doubtful.

It has been proved that sulysin plays a certain role in the pathogenesis of the infection because it is toxic to endothelial, epithelial and phagocytic cells (9, 12). After experimental inoculation of strains unable to synthesise sulysin, only mild symptoms of the disease were observed. Therefore, this protein does not seem to be critical for the virulence (1, 14). Moreover, it was also demonstrated that sulysin was not produced by all the isolates (15). This additionally confirms that sulysin cannot be considered as a universal marker of the virulence.

This study showed that less than half of the strains used in the experiment, isolated from pigs having the symptoms of streptococcosis, produced MRP, EF, and SLY together. Summarising the presented data, it can be concluded that the detection of the mentioned above proteins cannot be used to differentiate virulent and avirulent strains of S. suis, so further studies concerning its pathogenic properties are required.

References


